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SPONTANEOUS ATHEROSCLEROSIS IN PIGEONS AND THE ROLE OF OXIDATIVE METABOLISM

ROBERT FRANK SANTERRE

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BY

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B.S., Southern Connecticut State College, 1965

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ABSTRACT

SPONTANEOUS ATHEROSCLEROSIS IN PIGEONS
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From gross,histological, and histochemical observations of aortas from one- and six-year-old atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons the sequential development of atheromatous lesions at the celiac bifurcation was characterized. Lesions were found to develop specifically at sites of "cushion-like" smooth muscle cell aggregations in this region. Lesion development was shown to begin with sub-endothelial lipid accumulation; subsequent events including deeper lipid penetration, extracellular lipid accumulation, cell death, smooth muscle cell proliferation, and, finally, fibrosis and calcification result in progression of fatty streaks to advanced plaques. The role of hemodynamics in lesion localization is discussed. Based on a histological-histochemical grading scheme severity of lesions in six-year-old White Carneaux averaged two units greater than in Show Racers of the same age.

The celiac bifurcation was used as a model system

to compare respiration and oxidative phosphorylation in preatherosclerotic and diseased tissue and to determine whether there are differences in respiration and oxidative phosphorylation between susceptible, preatherosclerotic White Carneau and resistant Show Racer tissue. Oxygen consumption rates and respiratory control ratios were determined by manometric techniques. Preatherosclerotic White Carneaux had lower respiratory control ratios than corresponding Show Racers, and basal respiratory rates tended to be higher in the White Carneaux of this group.

These findings are discussed in terms of the relationship between oxidative energy production and atherosclerotic involvement. It is suggested the more tightly coupled oxidative phosphorylation in the atherosclerosis-resistant Show Racer may enhance the ability of this breed to withstand transient hypoxic stress.

INTRODUCTION

Despite more than seventy years of research atherosclerotic heart disease remains a major world health problem. In the United States alone more than 600,000 deaths annually are attributed to this disease, and the rate is rising steadily. Direct costs reach $3.1 billion annually; indirect costs have been placed at $4.3 billion. It has been estimated that some 23 million Americans are afflicted with the disease.

As stated by the World Health Organization atherosclerosis is:

> the variable combination of changes of the intima of arteries consisting of a focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue, and calcium deposits associated with medial changes.

Plasma lipid infiltration, hemodynamic stress, and mural thrombi have been implicated in the genesis of athero - sclerotic lesions (1,2,3). More recently, arterial wall metabolism has been implicated in atherogenesis (4), and the dynamics of energy metabolism in vascular smooth muscle cells has received special attention. Decreased oxidative phosphorylation in lesions, differences in respiratory rates between thoracic and abdominal portions of the aorta, and respiratory alterations in "preatherosclerotic" tissue have

been found (5,6). However, interpretation of the results is difficult because: (1) large areas of uninvolved tissue have been included in analyses diluting or masking changes in focally diseased regions; (2) many studies report only oxygen consumption rates and not P:O ratios or respiratory control ratios; (3) studies based on dry or wet weight are subject to variations due to large amounts of inert extracellular material in aortic tissue; and (4) findings in experimentally-induced atherosclerosis cannot easily be extrapolated to the spontaneous disease process.

Pigeons are susceptible to spontaneous atherosclerotic changes resembling the human disease. Smith, et al. (7) found that essential fatty acid deficient mitochondria of cultured aortic intimal cells from atherosclerosis-susceptible White Carneau (WC) pigeons had lower succinic dehydrogenase and mitochondrial ATPase activities than mitochondria from atherosclerosis-resistant Show Racer (SR) cells. Uncoupled oxidative phosphorylation was proposed as a contributing factor to lipid vacuole formation in cultured WC cells.

The purposes of this investigation were: (1) to compare respiration and oxidative phosphorylation in preatherosclerotic and diseased tissue, and (2) to determine whether there are differences in respiration and oxidative phosphorylation between susceptible WC and resistant SR

tissue. As a first step a detailed study of lesion development in WC and SR aortas was conducted. It was concluded that the development of atherosclerosis in the celiac bifurcation of the aorta is a predictable phenomenon in terms of histopathology, topography, age, and breed differences and that it should be possible to isolate metabolic changes associated with the spontaneous disease process in this model system. To this end, oxygen consumption rates and respiratory control ratios in muscular cushions from the celiac bifurcation of WC and SR aortas were determined by manometric techniques. Comparisons were made between breeds at three-five months, at two-three years, and on the basis of atherosclerotic involvement.

This thesis has been written in the form of two separate manuscripts. The first section describing lesion development will be submitted to American Journal of Pathology. The second section, an analysis of oxidative energy production, will be submitted to Atherosclerosis.

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SECTION I.

SPONTANEOUS ATHEROSCLEROSIS IN PIGEONS

A model system for study of metabolic parameters associated with atherogenesis

BY

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INTRODUCTION

Correlation of the many factors reportedly influencing atherosclerosis has proven difficult in experiments with intact animals. Diet, blood lipids, environmental stress, hemodynamics, alterations in arterial structure, and metabolism are but a few of the factors implicated in initiation of atheromatous changes. Moreover, interpretation of reports associating metabolic alterations with atherosclerosis has been hampered by the focal nature of the disease and by an inability to follow these alterations through transition stages in lesion development (1). In an effort to control these variables much research has been done with experimental atherosclerosis as a model system. However, spontaneous atherosclerosis has been shown to differ from the experimentally induced disease in many respects (2), and there are no well-defined model systems for study of the spontaneous process.

Clarkson, Lofland, Prichard, and co-workers (3-8) have described spontaneous aortic atherosclerosis in White Carneau pigeons and pointed out its close resemblance to the human disease. Cooke and Smith (9) subsequently described ultrastructural aspects of normal and diseased pigeon aortas. Differences in susceptibility between inbred strains of pigeons have been utilized to study relationships between

various metabolic patterns and atherosclerosis (10-12).

This communication extends previous work by describing the sequential development of atheromatous lesions in the celiac bifurcation of aortas from atherosclerosis-susceptible White Carneau and -resistant Show Racer pigeons. Lesion development is related to histology, topography, hemodynamics, and intimal thickening. Spontaneous atherogenesis in the celiac bifurcation is presented as a model system for the study of metabolic parameters in the arterial wall which may be associated with the disease.

MATERIALS AND METHODS

Subjects

Thirty White Carneau (WC) and 24 Show Racer (SR) pigeons were examined. The samples were divided approximately equally between one- and six-year-old birds with a few embryos and post-hatch squabs included. Sexes were equally represented. All birds were derived from inbred lines maintained by the Palmetto Pigeon Plant, Sumter, S.C. Most of the six-year-old birds had been housed in fly-coops, and the one-year-old birds were reared in battery cages with approximately two sq. ft. floor space per bird. In a separate study (unpublished) of the effects of environment on severity of atherosclerosis in WC and SR pigeons, it was found that the stress of prolonged or high-density battery-cage housing causes an increase in the rate of formation of severe lesions and tends to override the genetic resistance of the SR to the disease. This work will be reported at a later date. For purposes of the present communication possible increased severity in battery-caged, year-old birds had no significant effect on the comparisons made between them and six-year-old fly-coop birds.

Palmetto birds were fed a mixture of yellow corn, wheat, peas, kafir, and health grit. Our colonies were maintained on Purina pigeon pellets and similar health grit.

Light Microscopy

All birds were sacrificed by exsanguination. The entire aorta from the root down to, but usually not including the trifurcation was removed, washed in warm (37°C) buffered saline, pH 7.4, cleaned of excess connective tissue, and fixed at room temperature for 30-90 minutes in three percent phosphate-buffered glutaraldehyde, pH 7.4. After fixation, aortas were rinsed briefly in saline, immersed in Tissue-Tek O.C.T. Compound (Ames Company, Miles Laboratories, Inc., Elkhart, Indiana) and quick-frozen at -18°C. A serial survey of each aorta was made by cutting two or three 8 micron cross-sections every 700-800 microns; the sections were stained with hematoxylin-eosin, hematoxylin-Oil Red O (ORO), and sometimes Alcian blue-safranin O.

A severity index was obtained for each aorta by comparing hematoxylin-ORO stained sections with a series of six grades ranging from zero to five (see Figs. 7-11). The grading system was based on the following five criteria:

1. Amount of lipid present.
2. Extent of proliferation.
3. Amount of luminal circumference affected.
4. Degree of luminal occlusion.
5. Extent of necrosis, calcification, and vascularization.

Although the entire aorta was surveyed, the celiac

bifurcation demonstrated the most severe lesions and most important breed differences at six years. For this reason the detailed study of lesion development in this segment was undertaken.

Electron Microscopy

Advanced plaques from both breeds were fixed in three percent glutaraldehyde-0.1M cacodylate buffer, pH 7.3, washed in cacodylate-buffered six percent sucrose, post-fixed in two percent osmium-veronal, pH 7.3, dehydrated in a graded series of alcohols, and embedded in Epon. Adjacent thick (1 micron) and thin sections were cut on a Porter-Blum MT-2 ultramicrotome. Thin sections were stained with saturated uranyl acetate solution and Reynolds lead citrate, and examined with a Philips EM200 electron microscope.

RESULTS

Gross Observations

In the aortas of squabs, prior to the appearance of lipid, ridge-like thickenings are seen arising from the lateral edges of the celiac orifice and extending diagonally in a proximal direction (Fig. 1). At four-six months the earliest visible lipid accumulation appears as a fine white stippling on the surface of these cushions. As lesion development continues the lipid becomes yellow and the surface of the area is raised and may be rough or smooth. At later stages the main plaque, projecting well into the lumen, has a nodular, pearly appearance and is usually surrounded by a roughened, yellow skirt of involved tissue, sometimes fan-shaped and extensive in the proximal aspect (Fig. 2). In advanced lesions ulceration may be evident, particularly in centrally depressed regions of the largest plaques.

The topography of lesion development in the celiac bifurcation is quite specific (Fig. 3). In the young WC pigeon lesions appear earliest in the left lateral cushion lying below the ductus arteriosus. In older birds lesion development in the right lateral cushion progresses more rapidly, and, by six years, involvement is greatest in this cushion. No consistent differences in lesion development

between right and left cushions were seen in the SR. In both breeds involvement of areas surrounding the cushions progresses mainly upstream and laterally.

Histological and Ultrastructural Observations

A prominent feature of the celiac bifurcation in embryos and very young birds is the presence of paired muscular intimal thickenings corresponding to the ridge-like thickenings observed grossly (Fig. 4). The smooth muscle cells in these cushions are oriented longitudinally and the laminar organization of elastica present in the media is interrupted here (Fig. 5). The muscular nature of these cushions is emphasized because only in these smooth muscle cell aggregations does significant lipid accumulate leading to the production of advanced plaques.

The earliest evidence of atherosclerotic change in four- to six-month-old birds is the appearance of fine lipid droplets in the endothelium and subendothelial cells of the cushion (Fig. 6). Later fine lipid droplets become more prominent, appearing in the deeper regions of the cushion. Some proliferation of smooth muscle cells may also be seen at this time (Fig. 7). By one year this proliferative response can be a distinctive feature, and lipid involvement is frequently more extensive with some larger lipid pools (probably extracellular) accumulating (Fig. 8).

With further development, luminal protrusion of the lesion is amplified producing partial occlusion of the vessel. Much lipid is seen in large amorphous pools. Central necrosis is common, and fragmentation of surrounding medial elastic laminae may be found (Fig. 9). In this and later stages lesions expand not only luminally, but laterally and medially as well, by muscular transition of normal elastic areas.

Beyond this stage most lesions develop a fibrous cap. Ultrastructually the fibrous cap contains long, spindle-shaped, modified smooth muscle cells, foam cells, and prominent collagen bundles. Upper regions of the cap contain modified smooth muscle cells having few myofilaments with fusiform dense bodies, a patchy basement membrane envelope, typical pinocytotic vesicles along the cell membrane, numerous mitochondria, much granular endoplasmic reticulum, and many polysomes and free ribosomes (Fig. 13). Modified smooth muscle cells in deeper regions of the fibrous cap contain a similar array of organelles often including an active golgi apparatus. However, these cells contain many more myofilaments with fusiform and marginal dense bodies, as well as a distinct basement membrane envelope. Many of the cells contain darkly stained inclusions resembling material seen in the extracellular space (Fig. 14). In both regions the extracellular space contains little or no elastin, much collagen, and a large amount of vesiculated

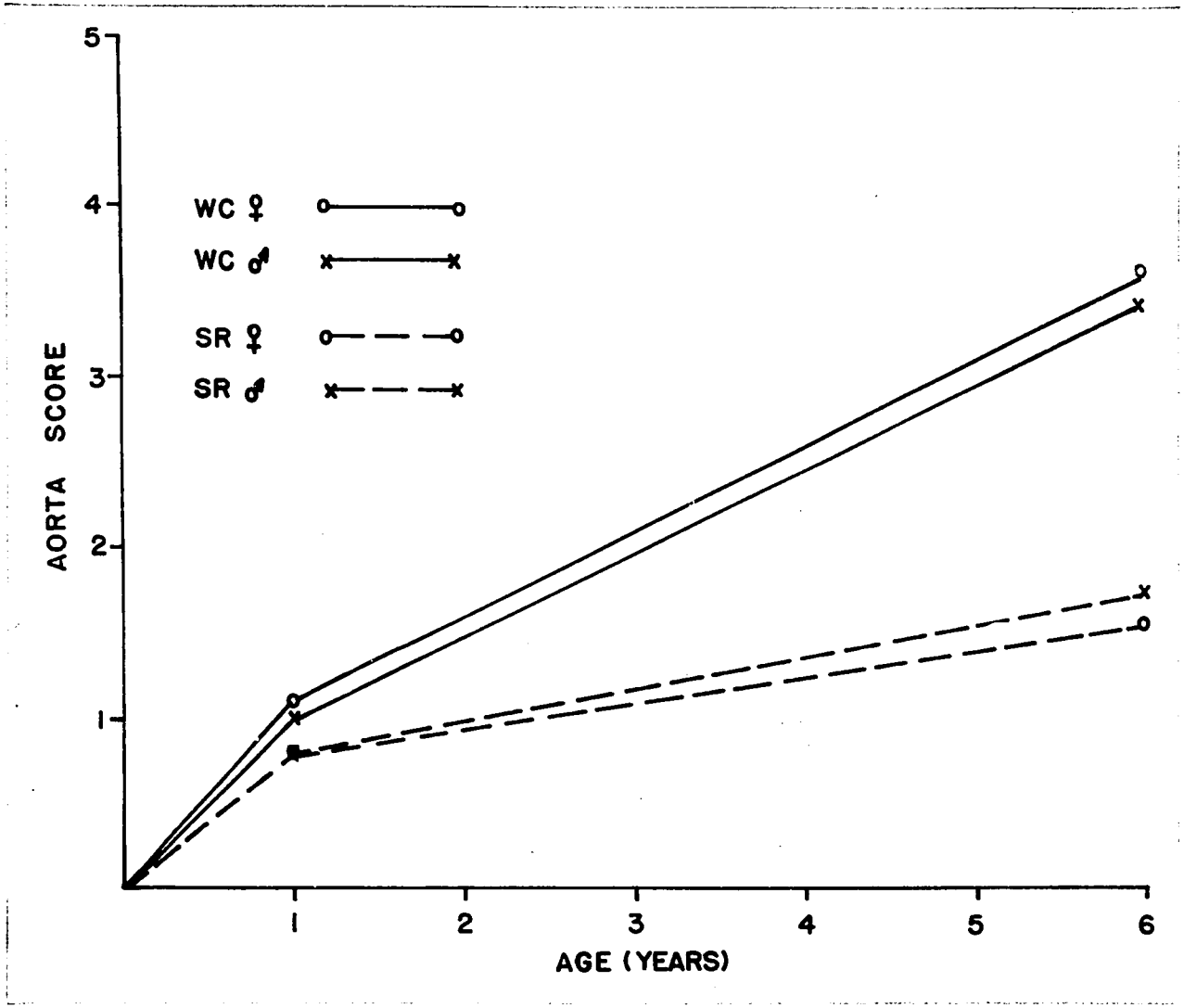
material perhaps representing cell debris and extracted lipid (Fig. 14).

In the deeper regions of the plaque cholesterol clefts are frequently found. There is usually significant luminal occlusion and medial thinning (Fig. 10). In the most advanced stages a variety of features are commonly encountered: massive fibrosis, sometimes nearly complete luminal occlusion, secondary necrosis and further lipid accumulation, vascularization, mineral deposits, ossification, and ulceration (Fig. 11) No thrombi and very few lymphocytes or macrophages were seen. Acid mucopolysaccharide accumulation is commonly seen in advanced plaques (Fig. 12).

Based on the severity index there was little difference between the two breeds at one year. However, at six years, involvement of the celiac bifurcation averaged two grades higher in the WC than in the SR (Text-fig. 1). Although 100 percent incidence of atherosclerosis of some degree was found in both breeds of pigeons at one and six years, only ten percent of the lesions in six-year-old Show Racers were above a grade 2 while more than sixty percent of the WC lesions were above this grade. The developmental history and histopathology of atherosclerotic plaques at a given stage were similar for both breeds.

While there were no significant differences in severity between sexes in either breed the sample size was too small to be conclusive. A trend toward higher grades in

Text-figure 1. Mean atherosclerotic grade in the celiac bifurcation of male and female White Carneau and Show Racer pigeons at one and six years.


AORTA SCORE
5
4
3
2
1
WC ♀
WC ♂
SR ♀
SR ♂
1
2
3
4
5
6
AGE (YEARS)

the WC female and the SR male was found at six years (Text-fig. 1).

DISCUSSION AND CONCLUSIONS

As McGill, et al. (1) pointed out, the lack of site-specific lesion development in humans has made it difficult to follow transition stages in atherosclerotic involvement. Our observations in the celiac bifurcation of the pigeon aorta show that fatty streaks can develop into fibrous plaques in a manner similar to that suggested for humans (13, 14).

Our findings are essentially similar to original reports on spontaneous atherosclerosis in pigeons by Clarkson, Lofland, Prichard, and co-workers (3-8). However, several important distinctions were noted. While they reported a difference in incidence of atherosclerosis between the two breeds (4,7), our birds exhibited 100 percent incidence of some degree of involvement in the celiac bifurcation at both one and six years. We found the major breed difference to be a much lower severity index in the SR than in the WC at six years. No differences were found in incidence, location, developmental history, or histopathology of WC and SR lesions as has been reported (4,7). These similarities suggest that the resistance of the SR may result from subtle metabolic factors.

The pattern of lesion localization and development at the celiac bifurcation is quite predictable. Similar findings have been reported for atherosclerosis in human

cerebral and coronary arteries (15,16) and for arteriosclerosis of the lower extremities (17). Hemodynamics undoubtedly plays a role in this localization as suggested by numerous workers (18-23). The work of Caro, et al. is most consistent with our observations. As predicted by their model we find atheroma localized lateral to the celiac orifice and progressing upstream, thus suggesting the presence of low shear rates and poor nutrition due to decreased exchange of materials in these regions. However, hemodynamics is probably not the sole factor. For example, it does not seem to explain the early involvement of the left lateral cushion and the subsequent faster progression and greater involvement of the right lateral cushion. It is possible that the cells in these areas have different inherent potentials (25,26).

Another very important factor, apparently acting synergistically with hemodynamics to localize lesion development, is the presence of raised muscular cushions representing a normal feature of vascular architecture at the celiac bifurcation and elsewhere (27-29). Muscular aggregations exhibit the earliest enzymatic changes associated with atherogenesis (30) and proliferative reactions induced by cholesterol feeding are also prominent there (31). In our system aggregations of smooth muscle cells seem to be a necessary prerequisite for lipid accumulation. Further work on the development of cushions and the role of blood flow in

vascular morphogenesis is needed.

The role of smooth muscle cells as the major cell type involved in the development of atherosclerotic lesions in a variety of animals including cows (32), swine (31), rabbits (33), baboons (34), humans (35), chickens (36), and pigeons (9) has been well documented. Lesions expand by a proliferative response of normal smooth muscle cells adjacent to regions of necrosis in a manner similar to that seen in the healing of arterial wounds (37). This study and others (9, 31-36, 38) suggest that modified smooth muscle cells found in early atheromatous lesions and even those found in fibrous caps of advanced lesions are very active metabolically. Such an interpretation is consistent with the presence of increased granular endoplasmic reticulum, many polysomal rosettes and free ribosomes, an active golgi apparatus, and numerous mitochondria in these cells. As Zemplenyi (30) has emphasized, the smooth muscle cell seems to dominate the metabolism of the normal arterial wall and of lesions at all stages of atherosclerotic involvement.

This study demonstrates that atherosclerosis in the celiac bifurcation is a predictable phenomenon in terms of histopathology, topography, age, and breed differences. It should be possible to isolate metabolic changes associated with the spontaneous disease process in this model system. A better understanding of the role of energy production, altered enzyme patterns, and acid mucopolysaccharide

metabolism is needed. Unequivocal answers to these questions and others concerning the role of metabolism in atherogenesis will be obtained only through the use of such a defined model system.

SUMMARY

The interpretation of metabolic studies related to early changes in spontaneous atherosclerosis has been hampered by the focal nature of the disease and by the lack of a well-defined model system of the disease process.

Gross, histological, and ultrastructural observations of lesion development at the celiac bifurcation of the aorta in atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons are compared and discussed in terms of hemodynamics, muscular aggregation, and altered smooth muscle cell metabolism.

Detailed knowledge of the morphological sequence of events in lesion formation and the predictability of lesion localization make the celiac bifurcation in White Carneau and Show Racer pigeons a useful model for making genetic comparisons of arterial wall metabolism and for investigating metabolic alterations occuring with atherogenesis.

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Figure 1. Non-involved aorta with ridge-like thickenings extending diagonally proximal from the celiac orifice (arrow). White Carneau male, 3-weeks-old. Unstained whole mount. X 2.6

Figure 2. Two large plaques on either side of the celiac orifice. Smaller plaques are also present at the renal branches and in the trifurcation. White Carneau female, 5-years-old. Unstained whole mount. X 2.1


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CENTIMETERS

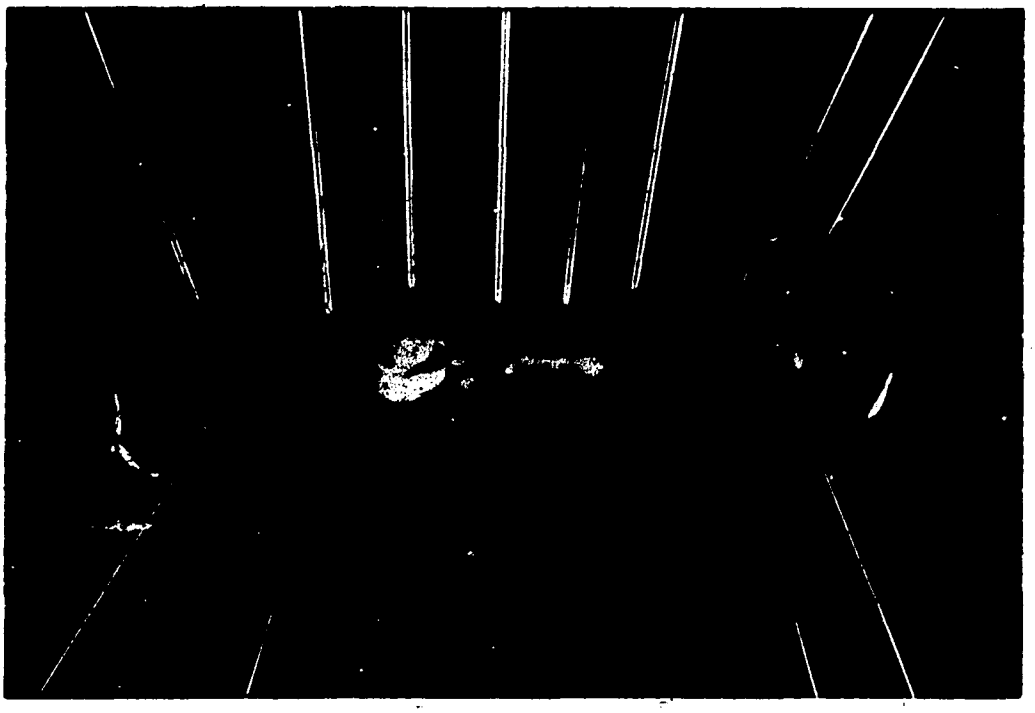


Figure 3. Diagrammatic representation of lesion progression in the cushions of the celiac orifice. In A and B the left cushion (lower) beneath the ductus arteriosus (d) is the most highly involved. The more rapid progression of the right cushion (upper) is depicted in C and D. Lesions progress mainly in the lateral and proximal direction (P). The extent of luminal occlusion can be followed in the cross-sectional diagrams. Cross-hatching represents proliferative growth with lipid accumulation.

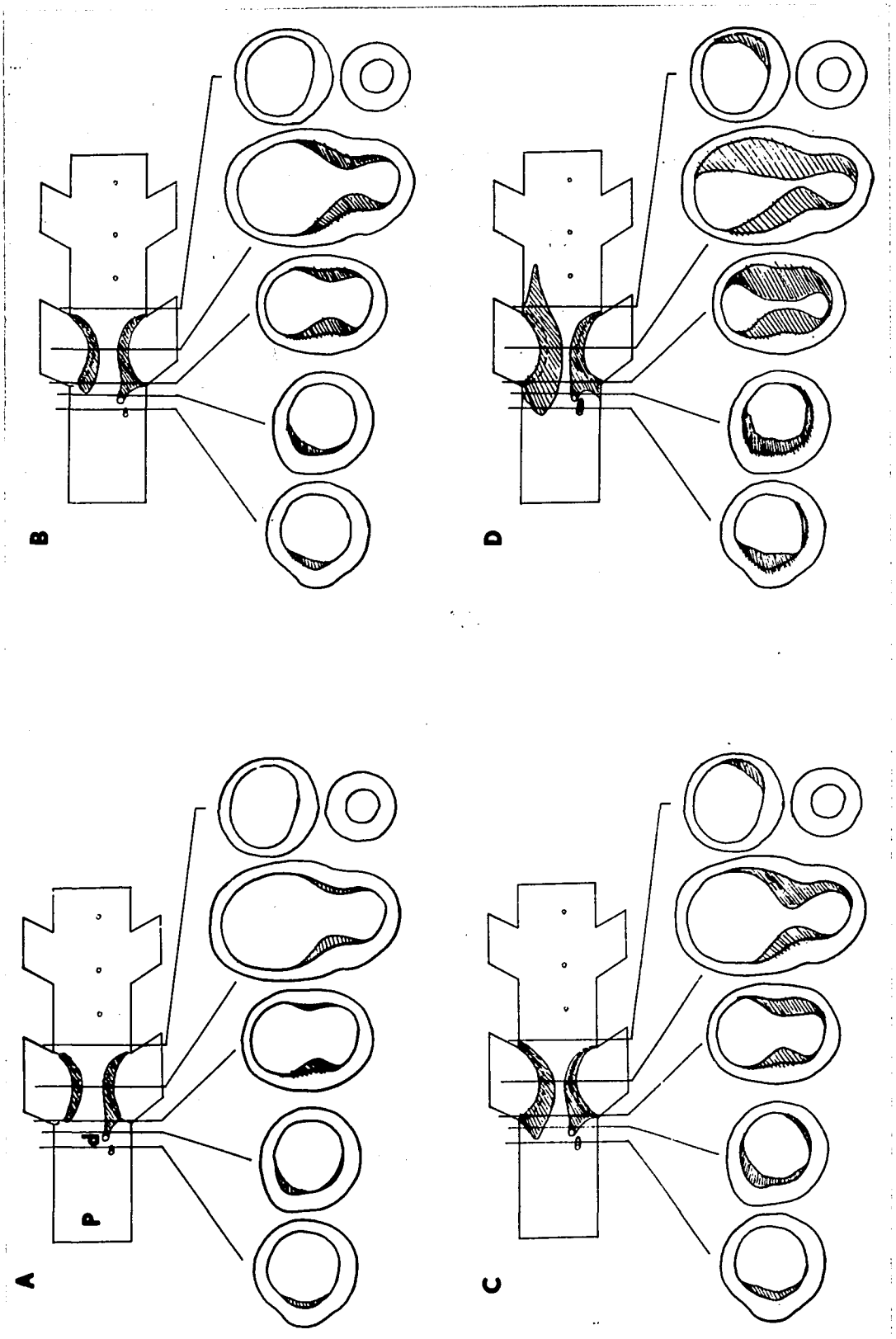

B
D
A
P
C

Figure 4. Cushions of smooth muscle cells protruding into the aortic lumen at the celiac bifurcation (arrows). White Carneau 10-day embryo. Hematoxylin-eosin. X 150

Figure 5. Normal cushion with longitudinally oriented smooth muscle cells. Laminar organization of elastic laminae at left (arrows) of figure is disrupted in the region of the cushion. Show Racer female, 4-weeks-old. Hematoxylin-eosin. X 330

Figure 6. Endothelial and subendothelial lipid deposition in an otherwise normal celiac cushion. White Carneau male, 3-months-old. Hematoxylin-Oil Red O. X 130

Figure 7. Grade 1 lesion. Many fine lipid droplets are present, some in deeper regions. Slight proliferation is visible. Show Racer male, 6-years-old. Hematoxylin-Oil Red O. X 130

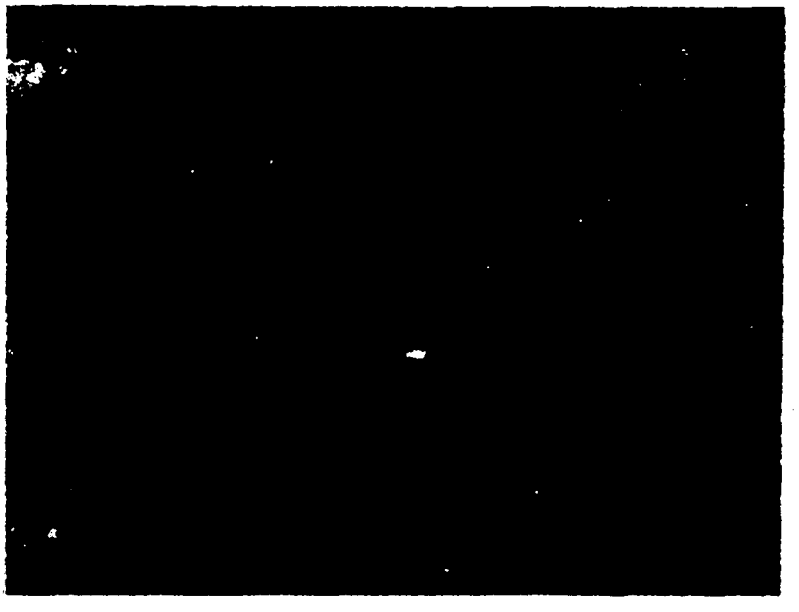
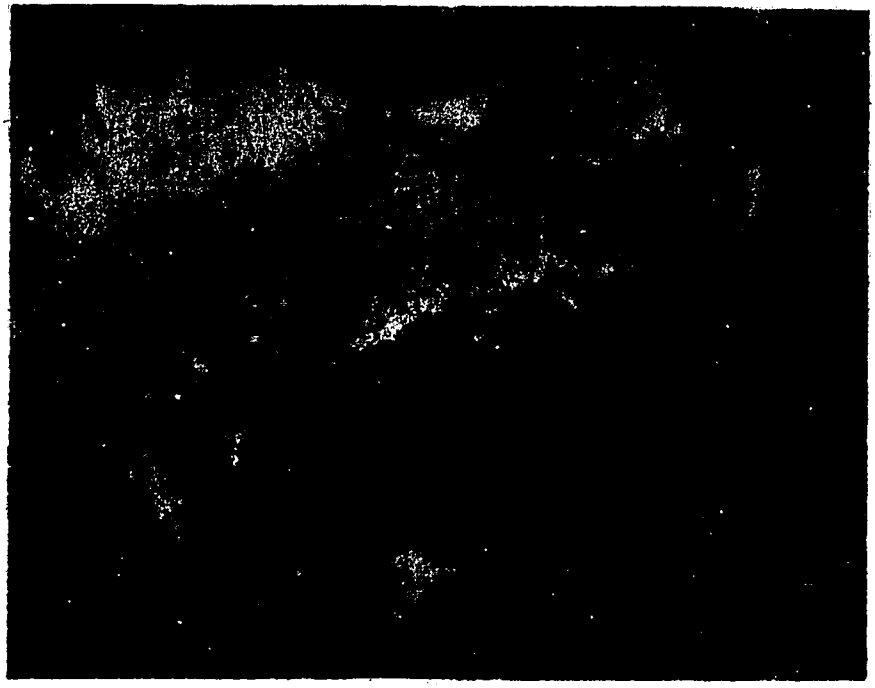


Figure 8. Grade 2 lesion. Much lipid accumulation, some in large pools. Marked proliferation has enlarged the cushion considerably. Show Racer female, 1-year-old. Hematoxylin-Oil Red O. X 130

Figure 9. Grade 3 lesion. Lipid-filled plaque exhibiting considerable luminal protrusion, central necrosis, and involvement of surrounding media. White Carneau female, 6-years-old. Hematoxylin-Oil Red O. X 45

Figure 10. Grade 4 lesion. A large fatty lesion with prominent fibrous cap, extensive central necrosis, cholesterol clefts, and medial thinning. White Carneau male, 6-years-old. Hematoxylin-Oil Red O. X 45

Figure 11. Grade 5 lesion. Massive fatty lesion nearly occluding entire lumen. Fibrous cap is thin; secondary necrosis and lipid accumulation in large amorphous pools is evident. Ulceration and mineral deposits are present. White Carneau female, 6-years-old. Hematoxylin-Oil Red O. X 45



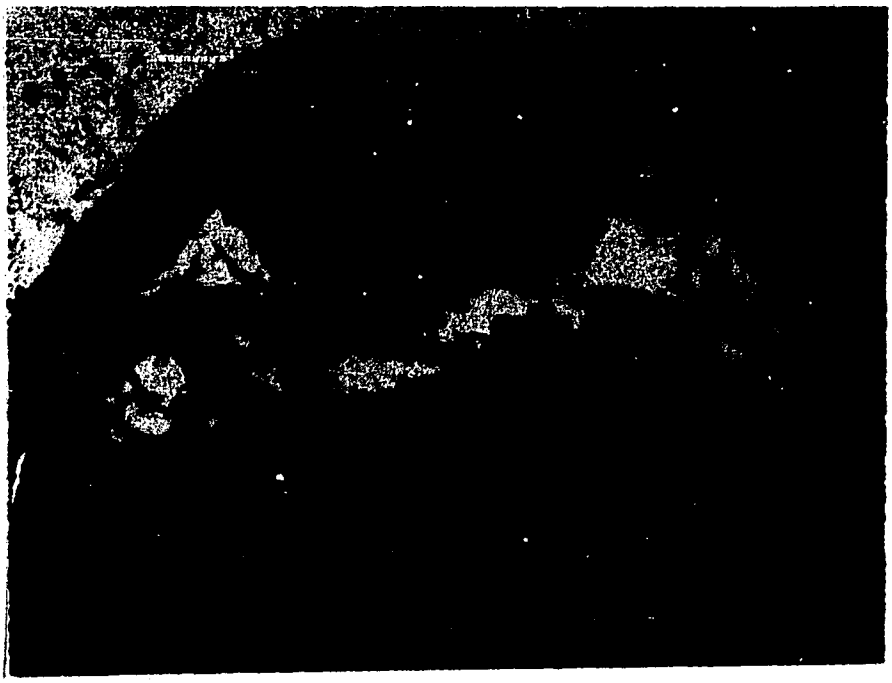


Figure 12. Acid mucopolysaccharide accumulation in the cap of an advanced lesion (arrows). White Carneau female, 6-years-old. Alcian blue-Safranin O. X 130

Figure 13. Modified smooth muscle cell in the upper region of the fibrous cap from an advanced lesion in a 7-year-old White Carneau female containing myofilaments (mf) with dense bodies (darts), much granular endoplasmic reticulum (GER), many mitochondria (M), and glycogen (G). Pinocytotic vesicles are prominent (arrows). The extracellular space contains fragmented basement membrane envelope (bme) and some collagen (C). Numerous vacuoles (V) containing material resembling that found in extracellular spaces are present. X 18,900

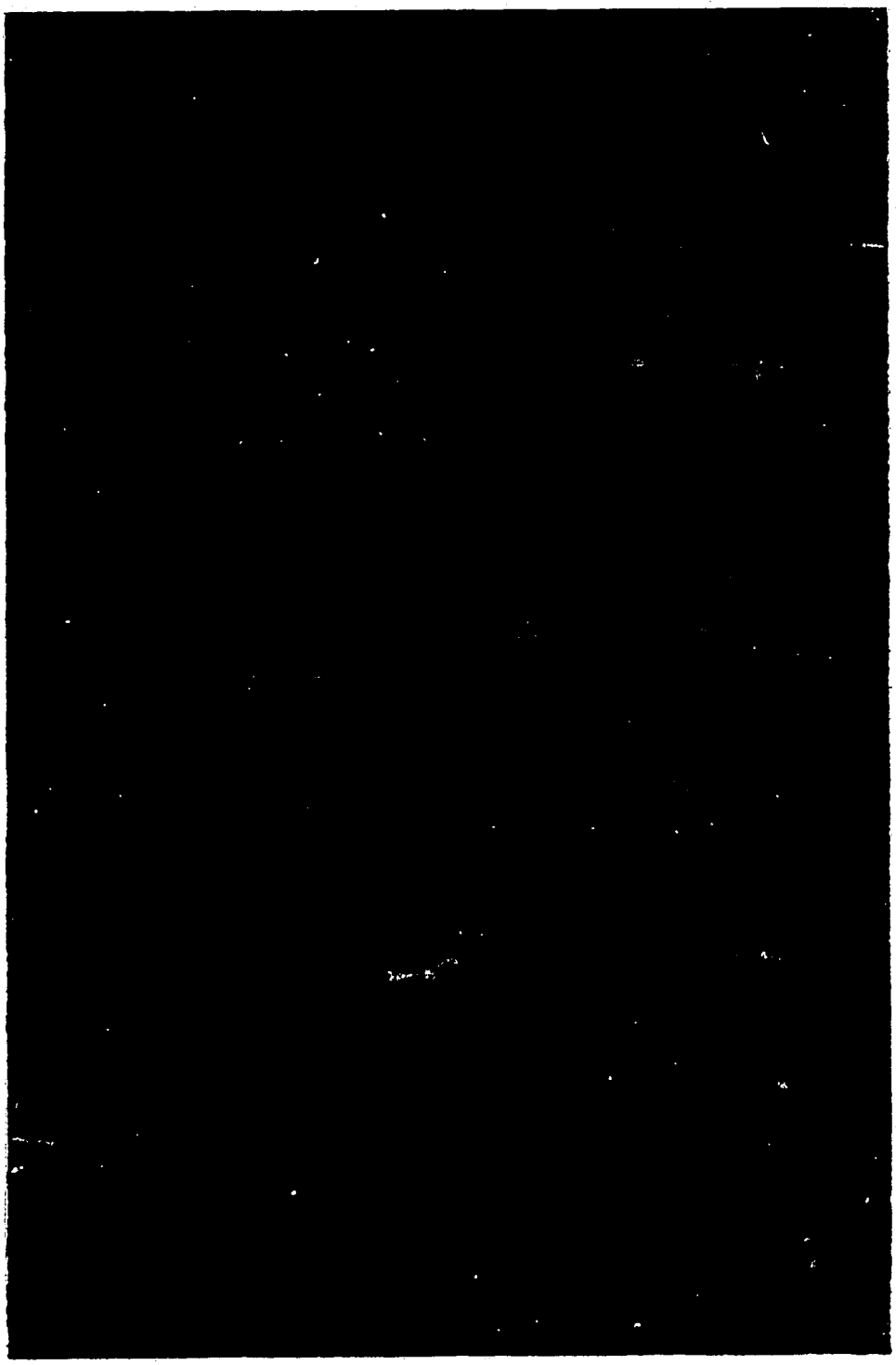
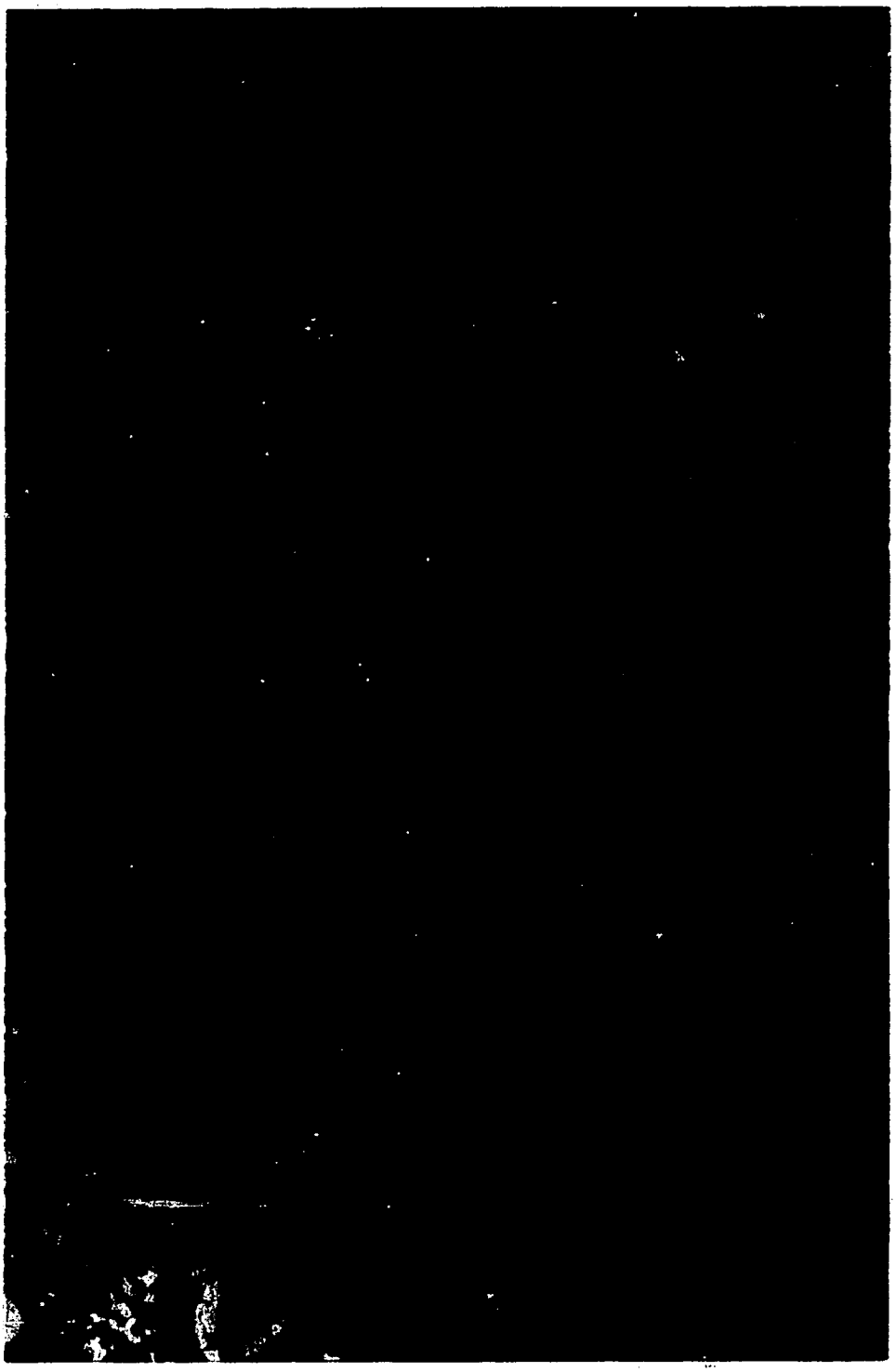


Figure 14. Deeper-lying region of the fibrous cap in Fig. 13. Modified smooth muscle cells in this region contain a similar array of organelles including an active golgi apparatus (*g*), many free ribosomes (R) and polysomes (p), and microtubules (small darts). These cells have many more myofilaments (mf) with dense bodies (large darts) and a distinct basement membrane envelope (bme). Collagen (C) is more prominent here. X 16,500



SECTION II.

DIFFERENCES IN RESPIRATORY CONTROL BETWEEN SUSCEPTIBLE, PREATHEROSCLEROTIC WHITE CARNEAU AND RESISTANT SHOW RACER PIGEONS

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ABSTRACT

Oxygen consumption rates and respiratory control ratios in muscular cushions from the celiac bifurcation of atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeon aortas were determined by manometric techniques. Comparisons were made between breeds at three-five months, at two-three years, and on the basis of atherosclerotic involvement. Preatherosclerotic White Carneau pigeons were found to have lower respiratory control ratios than corresponding Show Racers. The findings are discussed in terms of the relationship between oxidative energy production and atherosclerotic involvement. It is suggested that tighter coupling of oxidative phosphorylation in the Show Racer may enhance the ability of this breed to withstand transient hypoxic stress.

INTRODUCTION

In recent years many studies have been directed toward understanding the role of vascular smooth muscle metabolism in atherogenesis (see reviews of Adams, 1967; Whereat, 1967; Zemplenyi, 1968). Energy dynamics in the cell play a key role in regulating its metabolic functions, and emphasis has been placed on the role of glycolysis in vascular metabolism (Kirk, 1963). However, as Zemplenyi (1968) has pointed out, "if the utilization of glucose by oxidation is only 10 percent, nevertheless as much as 67.8 percent of the useful energy would be provided by this pathway", thus oxidative phosphorylation is a major energy source in vascular tissue. Many investigators have noted altered oxidative capacity in atherosclerotic aortas (Fischer and Geller, 1960; Munro et al., 1961; Whereat, 1964; Wolleman and Kocsar, 1964); a few have reported alterations in "preatherosclerotic" tissue (Maier and Haimovici, 1957; Scott et al., 1969). Interpretation of these results is difficult because: (1) large areas of uninvolved tissue have been included in the analyses diluting or masking changes present in focally diseased areas; (2) many studies report only oxygen consumption rates and not P:O ratios or respiratory control ratios; and (3) most of the respiration rates are based on dry or wet weight instead of DNA content which would eliminate variations due to large amounts of

inert extracellular material in aortic tissue. It remains unclear whether early respiratory alterations are caused by spontaneous atherosclerotic changes or vice versa.

In this communication oxygen consumption/hr/ug DNA and respiratory control ratios in celiac cushions (regions of predictable atherosclerotic involvement) were compared between atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons at both preatherosclerotic (three-five months) and involved stages (two-three years).

MATERIALS AND METHODS

White Carneau (WC) and Show Racer (SR) pigeons, obtained from the Palmetto Pigeon Plant, Sumter, S.C., were housed in fly coops and fed a mixture of yellow corn, wheat, peas, kafir, and Palmetto health grit. Aortic oxygen consumption and respiratory control ratios (RCR)[1] were compared between WC and SR pigeons in preatherosclerotic (three-five months) and atherosclerotic (two-three years) stages (Santerre et al., 1971). Some determinations were also made on embryos.

Birds were sacrificed by exsanguination and the aortas were quickly removed and placed in warm (37°C), oxygenated Hank's balanced salt solution (HBSS) as recommended by Somlyo and Somlyo (1968). The aortas were gently cleaned, dissected free of adventitia, opened longitudinally, and assigned an atherosclerotic grade (0-5) based on a visual estimate of the extent of involvement at the celiac bifurcation. Two pieces of aorta, 1 mm^2, were excised from the cushions of the celiac bifurcation or from

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(1) Chance and Hess (Science 129:700-708, 1959) have described the use of RCR's as a fine index of the integrity of oxidative phosphorylation in whole cells. The respiratory control ratio is defined as the ratio of state 3 (oxygen consumption in the presence of phosphate acceptor, ADP) to state 4 (oxygen consumption in the absence of ADP) respiration.

the upper-thoracic region and placed in a respiration chamber with 13 ul of incubation medium: HBSS made up to 26.7 mM substrate, 10 mM sodium fluoride, 2 mM iodoacetate (recrystallized 3x before use), and saturated with 100 percent oxygen. Substrates were succinate, pyruvate-malate, α-ketoglutarate, and glucose.

Oxygen consumption was measured in a constant-volume, mixing-type, ultramicrorespirometer by the "direct method of Warburg" (Umbreit et al., 1964). Construction of the manometric apparatus and mixing-type chambers has been described by Gregg (1947) and Gregg (1950, 1966). The 200 ul respiration chambers used in this study were made from our designs by Ace Glass Co., Vineland, N.J., and by Anderson Glass Co., Fitzwilliam, N.H. In our hands the apparatus was capable of measuring oxygen consumption rates as low as 0.01 ul per hour.

After establishing a steady respiration rate in the absence of phosphate acceptor (state 4), 2 ul of HBSS made up to 3.4 mM adenosine diphosphate (ADP) and 5.8 mM inorganic phosphate was added to the incubation medium and a state 3 respiration rate was established. In an attempt to control substrate level production of ADP, inhibition by ouabain and glucose-6-phosphate, was also tested; however, fluoride and iodoacetate gave best results. To verify respiratory control ratios adjacent pieces of tissue were sometimes incubated in the presence of an uncoupling agent,

3 mM 2,4-dinitrophenol (DNP).

Deoxyribonucleic acid (DNA) content of the tissue was determined by the Kissane-Robins ultramicrofluorometric method (Kissane and Robins, 1958) modified for sample sizes of 0.1-10 ug DNA and for use with a Turner filter fluorometer with high sensitivity optics and constant temperature sample holder.

Student's t-test for unpaired samples was utilized for all statistical analyses (Snedecor, 1967).

RESULTS

Respiratory control ratios in celiac cushions from preatherosclerotic (<grade 2) White Carneau pigeons were significantly lower ($P<0.05$) than corresponding values for Show Racers (Table I). State 4 respiration rates tended to be higher in the preatherosclerotic WC than in the SR; however, the differences were not significant, mainly due to high variability in the WC. No significant differences were found in respiratory rates or control ratios between breeds or sexes at either three-five months or two-three years. However, Table I shows that the SR has a consistently higher control ratio with but one noteworthy exception. The RCR for WC females at two-three years approaches the SR female and surpasses the SR male. These birds also show a relatively large increase in RCR from preatherosclerotic to diseased stages.

Among the various substrates tested, succinate gave the highest RCR's. With pyruvate-malate and α-ketoglutarate RCR's approaching 1.00 were consistently obtained. Glucose seemed to have an inhibitory effect yielding RCR's consistently below 1.00.

Almost all embryos tested, from both breeds, had RCR's of 1.00 or below, regardless of substrate used.

A few preliminary comparisons of RCR's were made between thoracic and celiac cushion areas in the same bird.

TABLE I. Comparison of respiratory rates and control ratios between breeds based on age and atherosclerotic grade.[a]

	WHITE CARNEAU			SHOW RACER		
		Oxygen Uptake			Oxygen Uptake	
	RCR	State 4	State 3	RCR	State 4	State 3
< grade 2	1.14 ± 0.08[b] (9)[c]	175 ± 21[e]	187 ± 20	1.46 ± 0.17 (13)	148 ± 13	196 ± 14
≥ grade 2	1.28 ± 0.14 (8)	159 ± 22	183 ± 11	1.36 ± 0.25 (3)	151 ± 15	203 ± 16
3-5 months						
male	1.18 ± 0.10 (6)	154 ± 18	168 ± 18	1.48 ± 0.17 (4)	143 ± 22	191 ± 26
female	1.16 ± 0.09 (4)	156 ± 28	174 ± 18	1.38 ± 0.19 (6)	148 ± 23	194 ± 24
2-3 years						
male	1.05 ± 0.06 (4)	207 ± 37	212 ± 35	1.28 ± 0.14 (5)	150 ± 10	186 ± 18
female	1.43 ± 0.19 (5)	145 ± 29	183 ± 12	1.46 ± 0.11 (5)	144 ± 16	190 ± 19

[a] succinate used as substrate.
[b] respiratory control ratio ± S.E.M.
[c] number of experiments in parentheses.
[d] difference between breeds significant at $P < 0.05$.
[e] respiratory rates, nanoliters O_2 /hr/ug DNA ± S.E.M.

RCR's for thoracic segments were about twice as high as normal celiac cushion segments in the WC. To determine whether the RCR's being measured were truly a function of coupled oxidative phosphorylation, tissue segments adjacent to the regular sampling area were sometimes incubated in the presence of an uncoupling agent (2, 4-dinitrophenol) simultaneously with the regular samples. With DNP, control ratios consistently fell to 1.00 or below. The drop in RCR appeared to be a result of stimulation of the state 4 respiration rate.

DISCUSSION

In this study the rate of oxygen uptake by pigeon aortic tissue ranged from 0.15-0.20 ul O_2/hr/ug DNA. Lofland and Clarkson (1965) reported that the ratio of DNA to dry weight in pigeon aortic tissue was 6 mg DNA/gm dry wt. Applying this conversion factor to our respiration rates, we obtained values of 0.9-1.2 ul O_2/hr/mg dry tissue, consistent with rates reported for aortic tissue in other species (Zemplenyi, 1968).

State 4 respiration rates in the preatherosclerotic WC tended to be higher than corresponding values for the SR. This was also true of cushion areas versus thoracic segments in the WC. Literature reports on respiration rates are quite variable. Increased respiration rates were found in diseased areas in cholesterol-fed swine aortas (Scott et al., 1969) and in atherosclerotic portions of rabbit aortas (Mandel et al., 1966; Whereat, 1961). However, Scott et al. (1967) reported no change in oxygen consumption of rat aortas in early stages of experimental atherosclerosis.

In rabbits, Fischer and Geller (1960) found that the aortic arch (most susceptible area) had the highest oxygen consumption. Christie and Dahl (1957) reported respiration rates in the susceptible abdominal aorta to be lower than those in the thoracic aorta in adult rats. However, Briggs et al. (1949) found no differences in rat aortas. The trend

toward higher state 4 respiration rates in the preatherosclerotic WC may reflect uncoupled oxidative phosphorylation, high endogenous levels of ADP, or reversed electron flow (Whereat, 1967).

Lamberg et al. (1968) reported low respiratory control ratios for mitochondria isolated from chick embryos at various stages of development. In the present study similar low RCR's were obtained from aortic segments of WC and SR embryos. Lamberg et al. (1968) suggested the low RCR's were caused by loose coupling of morphologically immature mitochondria. In pigeon embryos visual observation of a patent ductus arteriosus suggests oxygen levels are low in the prehatch bird. Closure of the ductus has been shown to require increased levels of oxygen normally supplied by the initiation of lung function (Hornblad, 1969). While embryonic pigeon mitochondria appear morphologically normal (Cooke and Smith, 1968), they may be functionally immature at this time when oxygen levels are low.

The relatively high RCR in two-three year-old WC females was an unexpected finding, particularly since the majority of these birds had atherosclerotic lesion grades of 4 or 5. However, as shown in previous studies modified smooth muscle cells in the fibrous caps of aortic lesions in pigeons (Santerre et al., 1971), and at sites of healing in injured dog aortas (Murray et al., 1966) appear metabolically active and may have more tightly coupled oxidative

phosphorylation.

When birds were grouped on the basis of atherosclerotic involvement the WC exhibited a significantly lower respiratory control ratio than the SR in the preatherosclerotic stage. While no significant differences in RCR's or respiratory rates between breeds as a function of age could be shown, there was a clear trend toward lowered RCR's in both the male and female WC; lack of significance was probably due to high variability and small sample sizes.

The reasons for this lowered respiratory control ratio are not clear. As previously pointed out, uncoupled oxidative phosphorylation, high endogenous levels of ADP, or reversed electron flow could be the cause. In addition, Smith et al. (1966) found that cultured aortic intimal cells from WC pigeons were deficient in essential fatty acids (EFA) and had lower mitochondrial ATP-ase activity when compared to cells from SR pigeons. Young (1969) has reported that serum sterol esters in young WC's were also EFA deficient in comparison to those in young SR's. Ito and Johnson (1964) found a low RCR for liver mitochondria from EFA deficient rats. It has been suggested that such damaged mitochondria may accumulate lipid and contribute to early atherogenesis (Murray et al., 1968; Smith et al., 1966). Fatty acids, which could arise from this lipid are potent uncouplers of oxidative phosphorylation (Vasquez-Colon et al., 1966).

The possibility of a genetic defect in the phosphory-

lating mechanism must also be considered. Lochner et al. (1968) have described such a biochemical lesion in a strain of hereditary myopathic hamsters. Sarcosomes from this myopathic heart tissue had a significantly lower RCR.

Impaired oxidative phosphorylation could be further aggravated by the marginally hypoxic conditions existing in the inner third of the aortic wall (Zemplenyi, 1968). Intimal thickening and lesion development would lead to increased hypoxia. Hypoxia can cause decreases in oxygen consumption, phosphorylation, and RCR's (Jennings et al., 1967; Lindenmayer et al., 1968), as well as decrease levels of oxidative enzymes and increase levels of glycolytic enzymes (Bartley et al., 1968). Lazzarini-Robertson (1968) found permeability to extracellular lipids to be increased in human intimal cells cultured under low oxygen tension. Hypoxia has also been shown to stimulate fibrogenesis, especially synthesis of sulfated mucopolysaccharides and collagen (Helin et al., 1969). Summarizing the biochemical derangements associated with hypoxia, McDowall (1969) emphasized reduced transport, increased NADH:NAD^+ ratios, ineffectiveness of the Krebs cycle, and diversion of pyruvate to lactate in an attempt to maintain ATP levels until the final stages of anoxia.

Whereat (1967) has suggested that succinate, via reversed electron flow, controls the NADH:NAD^+ ratio and plays a major role in controlling respiration and synthesis.

Zemplenyi (1968) has reported adaptation of lactate dehydrogenase (LDH) isozymes to more active forms under hypoxic conditions. Reversed electron flow may represent a peculiar adaptation of vascular tissue to the transient hypoxic conditions it faces. During periods of local hypoxia high levels of NADH could be oxidized by LDH, continually replenishing NAD^+ needed for oxidative steps in substrate level phosphorylation.

Nevertheless, chronic hypoxia would eventually result in an energy deficit, increased lipid uptake, and decreased lipid dispersal. The more tightly coupled oxidative phosphorylation found in SR's may enable vascular smooth muscle cells in these birds to maintain a lower NADH:NAD^+ ratio and higher levels of oxidatively formed ATP, giving them a greater ability to disperse lipids during periods of transient hypoxia.

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